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Award Number: W81XWH-14-1-0144

TITLE: Accelerate Genomic Aging in Congenital Neutropenia

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REPORT DATE: August 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2015	Annual	15 Jul 2014-14 Jul 2015

4. TITLE AND SUBTITLE
Accelerate Genomic Aging in Congenital Neutropenia

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0144

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
.
Washington University
St. Louis, MO 63130

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Transformation to a myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) is perhaps the major clinical concern in patients with severe congenital neutropenia (SCN) and Shwachman-Diamond syndrome (SDS), with a cumulative risk of MDS/AML of more than 20%. The prognosis of patients with SCN or SDS who develop MDS/AML is poor and treatment options are limited. However, the molecular mechanisms contributing to transformation to MDS/AML in these disorders are poorly understand, limiting the development of new therapies or strategies for risk stratification or early detection. In this proposal we test the novel hypothesis that premature genomic aging of hematopoietic stem cells (HSCs) in patients with SCN and SDS contributes to the high rate of transformation to MDS/AML. Each time an HSC divides, it has a chance of acquiring a new mutation. Thus, the number of mutations in an HSC provides an estimate of its genomic age. We have developed a novel assay to measure the mutation burden in HSCs using either blood or bone marrow. Using this assay, we published a paper showing that mutations accumulate in HSCs as a function of age and likely accounts for the increased incidence of AML/MDS in the elderly. It follows, that conditions that increase the rate with which HSCs accumulate mutations will result in an increased incidence of AML/MDS and presentation at an earlier age.

15. SUBJECT TERMS-

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU		19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. INTRODUCTION

The goal of this research is to define the molecular mechanisms responsible for the markedly increased risk of transformation to myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) in patients with congenital neutropenia. We hypothesize that replicative stress and/or changes in the bone marrow microenvironment in patients with congenital neutropenia leads to a higher rate of accumulation of mutations in hematopoietic stem/progenitor cells (HSPCs), which, in turn, contributes to transformation to MDS/AML. We further hypothesize that G-CSF treatment accelerates the accumulation of mutations in HSPCs. Finally, we hypothesize that truncation mutations of *CSF3R*, which are common in patients with severe congenital neutropenia (SCN) and are associated with increased G-CSF signaling and transformation to MDS/AML, accentuate the rate of mutation accumulation. We will test these hypotheses in the following Specific Aims. Aim 1. To determine whether HSPCs undergo premature genomic aging in SCN or SDS. We will measure the mutation burden in individual HSPCs from patients with SCN, Shwachman-Diamond syndrome (SDS), cyclic neutropenia, or age-matched healthy controls. Aim 2. To determine whether increased G-CSF signaling accelerates the mutation rate in HSPCs. Here, we will assess the impact of prolonged (6 month) G-CSF therapy on HPSC mutation burden in mice. These data will provide novel insight into the mechanisms of leukemic transformation in CN. They also should provide new insight into the safety of long-term G-CSF therapy in CN. Finally, our novel assay to measure mutation burden in HSPCs may provide an approach to assess DNA damage after exposure to genotoxic agents, such as radiation.

2. KEYWORDS

Congenital neutropenia
Severe congenital neutropenia
Shwachman Diamond syndrome
Cyclic neutropenia
Hematopoietic stem cells
Granulocyte colony-stimulating factor
Granulocyte colony-stimulating factor receptor
Acute myeloid leukemia
Myelodysplastic syndrome

3. ACCOMPLISHMENTS

The major goals and objectives of this research remain the same as originally proposed. Progress and plans for each of the tasks proposed in the original Statement of Work are detailed below.

Task 1. To determine whether HSCs undergo premature genomic aging in congenital neutropenia (Timeframe: 1-32 months). In this task, we will assess the mutation burden in individual HSPC clones obtained from healthy controls or patients with congenital neutropenia. As detailed in task 1d, we have had unanticipated difficulties in expanding HSPCs for

sequencing. *This problem has been resolved, but the resulting delay has extended our timeframe for completing this task from 24 months to 32 month*s.

1a. Obtain human studies approval for whole exome sequencing of healthy controls (**Timeframe 1-2 months; completed**). Human studies approval at Washington University, University of Michigan, and the University of Washington have been obtained.

1b. Obtain human studies approval for studies from the DoD Human Research Projection Office (**Time frame 1-3 months; completed**). DoD approval has been obtained.

1c. Obtain human blood or bone marrow samples from patients with SDS, SCN, cyclic neutropenia, or healthy controls (**Timeframe 3-24 months, ongoing**). We proposed to obtain a total of 15 bone marrow of blood samples each from patients SDS, SCN, cyclic neutropenia, or age-matched healthy controls. Table 1 shows our progress to date. We have meet or exceeded our original goals for SCN and healthy controls, are one short for SDS, and still need 6 cyclic neutropenia samples. As outlined in the "task 1d" section, our success rate in obtaining suitable HSPC colonies for sequencing has been less than expected. *Thus, we have increased our target sample number to 20 for SDS and SCN and 30 for healthy controls, and we have extended the timeframe to achieve this objective from 18 months to 24.*

Table 1. Human HSPC expansion

Sample type	Total # of samples	Successful HSPC expansion	Failed HSPC expansion	HSPC expansion in process	Frozen Samples Remaining
Normal	20	5	11	4	0
SCN	17	8	5	1	3
SDS	13	1	2	2	8
Cyclic	9	3	3	0	3

The total number of blood or bone marrow samples obtained to date is shown. The number of successful and failed HSPC expansions (defined as generating at least 4 large HSPC colonies) are shown. In some cases, we have cryopreserved (frozen) bone marrow available for HSPC expansion

1d. Generate hematopoietic stem/progenitor cell (HSPC) colonies from patients with CN or healthy controls (**Timeframe 3-24 months; ongoing**). Our progress to date is summarized in Table 1. We encountered some technical difficulty in expanding HSPC colonies. This necessitated further optimization of the culture conditions, as follows: 1) we have reduced cell attrition during sorting by reducing the sample flow and sheer rate; 2) we have optimized the HPSC sorting strategy; and 3) we have optimized stromal cell viability. Figure 1 shows our success rate before and after optimization of our culture conditions. Our current success rate is now approximately 88%. Thus, with the increased number of human samples (task 1c), we anticipate no difficulty in achieving this objective. *However, we have extended our timeframe to complete this task from 18 months to 24 months.*

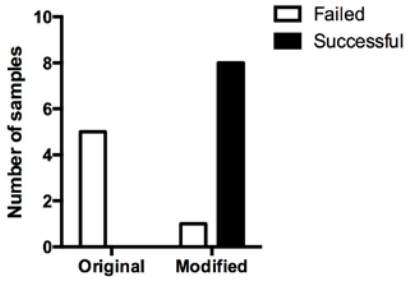

Figure 1. HSPC expansion. Shown is the number of failed and successful HSPC expansions for human SCN samples using the original or modified expansion protocol.

1e. Sequence HSPC clones and bone marrow fibroblasts (**Timeframe 12-27 months, planned**). The number of samples that have successfully undergone HSPC expansion and DNA isolation is summarized in Table 1. Data quality and cost effectiveness are better if the samples are sequenced in batches. We anticipate sequencing the first set of samples in the next 1 month. *Due to the difficulty in expanding HSPC clones (task 1d), we are extending the timeline to complete this task from 21 to 28 months*.

1f. Analysis of the sequence data (**Timeframe 15-28 months, planned**). It should take approximately 4 weeks to analyze the data. *Due to the difficulty in expanding HSPC clones (task 1d), we are extending the timeline to complete this task from 21 to 28 months.*

1g. Validation of mutations (**Timeframe 18-32 months, planned**). All somatic mutations identified by sequencing will be validated by generating custom capture reagents and sequencing the HSPC and bone marrow fibroblast DNA again. This takes approximately 3 months. *Due to the difficulty in expanding HSPC clones (task 1d), we are extending the timeline to complete this task from 24 to 32 months.*

Task 2. To determine whether increased G-CSF signaling accelerates the mutation rate in HSPCs (Timeframe 1-21 months). In this task, we will assess the mutation burden in individual murine HSPC clones exposed to G-CSF treatment for 6 months. These analyses will be performed in wildtype and *Csf3r* mutant mice. As detailed below, we are on schedule with this task, and we anticipate generating the first set of sequencing data in the next few months.

2a. Obtain regulatory approval from the DOD Animal Care and Use Review Office (**Timeframe 2 months, completed**). DoD Animal Care approval has been obtained. This task is complete.

2c. Generate HSPC colonies from mice (**Timeframe 5-14 months, ongoing**). We have successfully generated HSPC colonies from 27 wildtype or *Csf3r* mutant mice treated with G-CSF or saline alone for 6 months (Table 2).

Table 2. Murine HSPC expansion

Sample type	G-CSF treatment	Number of mice	Successful HSPC expansion	Failed HSPC expansion	Frozen Samples Remaining
Wildtype	No	12	9	0	3
Wildtype	Yes	12	9	0	3
Csf3r mutant	No	12	4	0	8
Csf3r mutant	Yes	12	5	1	6

Wildtype or Csf3r mutant mice were treated with saline alone of G-CSF for 6 months (n = 12, each cohort). Shown are the number of successful and failed HSPC expansions (defined as generating at least 4 large HSPC colonies).
In some cases, we have cryopreserved (frozen) bone marrow available for HSPC expansion

2d. Sequence HSPC clones (**Timeframe: 12-16 months, ongoing**). We recently submitted for sequencing DNA isolated from HSPC clones from a total of 22 mice. Sequencing libraries have been generated and these samples are in the queue for sequencing, which we anticipate will be completed in the next 1 month. We intend to submit HSPC clone DNA from the remaining 18 mice, once we verify the quality of the first round of sequencing. We anticipate meeting the original timeframe to complete this task of 16 months.

2e. Analysis of the sequence data (**Timeframe 14-18 months, planned**). We are on schedule to complete this task by 18 months.

2f. Validation of mutations (**Timeframe 14-21 months, planned**). All somatic mutations identified by sequencing will be validated by generating custom capture reagents and sequencing the HSPC and bone marrow fibroblast DNA again. This takes approximately 3 months. We are on schedule to complete this task by 21 months.

Opportunities for training and professional development: nothing to report

Dissemination of results to communities of interest: nothing to report

Plans for the next reporting cycle. With minor exceptions detailed in Task 1, we are on schedule to complete the goals of the original proposal. Detailed plans and a timeframe for each of the tasks of our original proposal are outlined above.

4. IMPACT

Impact on the development of the principal discipline: We have optimized the approach to culture and expand both human and murine HSPCs clones. This technique will be of use to investigators in the field studying clonal architecture and clonal evolution in hematopoiesis.

Impact on other disciplines: nothing to report

Impact on technology transfer: nothing to report

Impact on society: nothing to report

5. CHANGES/PROBLEMS

Changes in approach: nothing to report

Actual or anticipated problems: As outline in section 2, we experienced unanticipated difficulties in efficiently expanding individual human HSPC clones . This problem has been resolved, and we anticipate no difficulty in achieving the original objectives of this proposal. However, this problem has resulted in a significant delay, and we now extend the timeframe to complete this research from 24 months to 32 months.

Significant changes in the use or care of human subjects, vertebrate animals, biohazards, and/or select agents: nothing to report

6. PRODUCTS

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals worked on this project:

Name:	Daniel C. Link
Project Role:	Principal Investigator
Researcher Identifier:	0000-0002-3410-3580
Nearest person month involved:	1 month
Contribution to Project:	no change
Funding Support:	no change

Name:	Jun Xia
Project Role:	Staff Scientist
Researcher Identifier:	0000-0003-0946-4082
Nearest person month involved:	3 months
Contribution to Project:	no change
Funding Support:	no change

Changes in active or other support of the PD/PI:

1R01CA194552-01 (DiPersio, PI) 04/01/15-03/31/20
NIH/NCI $300.000
Title: Retargeting Agents to Treat AML
This is a recently awarded grant on which I serve as a collaborator (0.20 CAL). There is no overlap with the DoD Proposal.

TRA 6030-10 (Link, PI) 10/1/09-9/30/2013 2.00 CAL
Leukemia & Lymphoma Society $180,000
Mutational Profiling of microRNAs in t-AML/t-MDS
This grant is completed

RO1 CA136617 (Link, PI) 5/1/09-4/31/14 1.80 CAL
National Institutes of Health $250,000
Clonal Dominance of Hematopoietic Stem Cells Expressing Mutant CSF3R
This grant is completed

7539-55 (Link, PI) 1/1/2012-12/30/2013 0.60 CAL

Barnes-Jewish Hospital Foundation, Cancer Frontier Grant $100,000
Targeting the bone marrow microenvironment in multiple myeloma
This grant is completed

What other organizations are involved as partners:

Organization Name: The University of Washington
Location of Organization: Seattle, Washington
Partner's contribution to the project: Drs. Dale and Shimamura provide coded human blood or bone marrow samples from patients with congenital neutropenia.
No changes

Organization Name: University of Michigan
Location of Organization: Ann Arbor, MI
Partner's contribution to the project: Dr. Larry Boxer provide coded human blood or bone marrow samples from patients with congenital neutropenia.
No changes

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES: None